



# Chloroplast protein synthesis elongation factor, EF-Tu, reduces thermal aggregation of rubisco activase

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## Summary

Chloroplast protein synthesis elongation factor, EF-Tu, has been implicated in heat tolerance in maize. The recombinant precursor of this protein, pre-EF-Tu, has been found to exhibit chaperone activity and protect heat-labile proteins, such as citrate synthase and malate dehydrogenase, from thermal aggregation. Chloroplast EF-Tu is highly conserved and it is possible that the chaperone activity of this protein is not species-specific. In this study, we investigated the effect of native wheat pre-EF-Tu on thermal aggregation of rubisco activase. Additionally, we investigated the effect of native and recombinant maize pre-EF-Tu on activase aggregation. Activase was chosen because it displays an exceptional sensitivity to thermal aggregation and constrains photosynthesis at high temperature. The native precursors of both wheat and maize EF-Tu displayed chaperone activity, as shown by the capacity of both proteins to reduce thermal aggregation of rubisco activase *in vitro*. Similarly, the recombinant maize pre-EF-Tu protected activase from thermal aggregation. This is the first report on chaperone activity of native pre-EF-Tu and the first evidence for thermal protection of a photosynthetic enzyme by this putative chaperone. The

**Abbreviations:** PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; SDS, sodium dodecyl sulfate

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results are consistent with the hypothesis that chloroplast EF-Tu plays a functional role in heat tolerance by acting as a molecular chaperone.

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## Introduction

High temperatures or heat stress adversely affects plant cells, causing denaturation and aggregation of most proteins (Levitt, 1980) and damage to cellular membranes (Armond et al., 1980; Levitt, 1980; Ristic and Cass, 1992, 1993). Heat stress also affects cell metabolism, causing changes in the rates of many biochemical reactions (Berry and Björkman, 1980; Levitt, 1980). High temperatures reduce photosystem II activity, photophosphorylation, photosynthetic enzyme activity, dark respiration (Berry and Björkman, 1980), protein synthesis, and ion uptake (Levitt, 1980). Of all the metabolic processes that are affected by heat stress, the one that appears to be most sensitive to inhibition is CO<sub>2</sub> fixation (Berry and Björkman, 1980).

The inhibition of CO<sub>2</sub> fixation during heat stress is primarily caused by inactivation of rubisco activase (Feller et al., 1998; Salvucci et al., 2001). Rubisco activase is a nuclear-encoded, soluble chloroplast enzyme that regulates the activity of rubisco (Andrews, 1996; Portis, 2002; Spreitzer and Salvucci, 2002). In most species studied, activase is found in two isoforms, the longer  $\alpha$  (43–46 kDa) and the shorter  $\beta$  (41–42 kDa), both of which are capable of promoting rubisco activation (Shen et al., 1991). Activase is highly sensitive to heat stress, as it loses its activity at moderately high temperatures (Crafts-Brandner et al., 1997; Feller et al., 1998). Loss of activase activity during heat stress is attributed to an exceptional sensitivity of this protein to heat denaturation and aggregation (Salvucci et al., 2001).

Plants cells have evolved several mechanisms that enable them to alleviate the negative effects of heat stress (Levitt, 1980). One such mechanism is the synthesis of heat-shock proteins (HSPs) (Vierling, 1991; Schöffl et al., 1998; Feder and Hofmann, 1999; Maestri et al., 2002). HSPs play a central role in heat tolerance by acting as molecular chaperones; that is, they bind and stabilize heat-labile proteins, protecting them from thermal aggregation (Vierling, 1991; Hendrick and Hartl, 1993; Feder and Hofmann, 1999; Lee and Vierling, 2000; Basha et al., 2004).

Studies have shown that some other proteins, in addition to HSPs, play a role in heat tolerance by acting as molecular chaperones (Caldas et al.,

1998, 2000; Rao et al., 2004). Examples include the prokaryotic protein synthesis initiation factor IF2, protein synthesis elongation factors EF-G (Caldas et al., 2000) and EF-Tu (Caldas et al., 1998; Malki et al., 2002), and the mammalian mitochondrial translation elongation factor, EF-Tu-mt (Suzuki et al., 2007). These proteins perform a chaperone function by interacting with unfolded and denatured proteins, thereby protecting them from thermal aggregation.

Recent studies have suggested that maize (*Zea mays*) chloroplast protein synthesis elongation factor, EF-Tu, plays a role in heat tolerance (Ristic et al., 2004; Momcilovic and Ristic, 2004) by acting as a molecular chaperone (Rao et al., 2004). The recombinant precursor of this protein, pre-EF-Tu, was found to protect heat-labile citrate synthase and malate dehydrogenase from thermal aggregation (Rao et al., 2004). Chloroplast EF-Tu is highly conserved (Baldauf and Palmer, 1990; Ursin et al., 1993; Sugita et al., 1994; Maurer et al., 1996; Lee et al., 1997; Bhadula et al., 2001), and it is possible that EF-Tu from other species also displays chaperone activity. In this study, we examined the effect of native pre-EF-Tu from wheat (*Triticum aestivum*) on thermal aggregation of rubisco activase. We also examined the influence of native and recombinant maize pre-EF-Tu on activase aggregation. Rubisco activase was chosen because it is the major protein that denatures/aggregates (Salvucci et al., 2001) and constrains photosynthesis at high temperature (Crafts-Brandner and Salvucci, 2000).

## Materials and methods

### Materials

We used recombinant maize (*Z. mays* L.) rubisco activase, the native wheat (*T. aestivum* L.) and maize precursor of chloroplast EF-Tu (pre-EF-Tu), and the recombinant proteins that display chaperone activity, maize pre-EF-Tu (Rao et al., 2004), and *Escherichia coli* DnaK (Diamant et al., 2000). Recombinant maize rubisco activase was expressed in, and purified from, *E. coli* BL-21 (DE3). Native wheat and maize pre-EF-Tu were isolated and purified from the leaf tissue of spring wheat cultivar Seri-82 and maize line B-73, respectively. Recombinant maize pre-EF-Tu was previously isolated and purified from *E. coli* strain DH5 $\alpha$  (Rao et al., 2004), and in the current study we purified additional amounts

of this protein. Recombinant DnaK was purchased from Nventa Biopharmaceuticals (San Diego, CA).

### Cloning and expression of rubisco activase from maize

A full-length cDNA encoding maize ribulose 1,5-bisphosphate carboxylase/oxygenase activase (*Zmrc1*, GenBank accession no. [AF084478](#)) was synthesized using the ThermoScript reverse transcription (RT-PCR) system according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA). RNA was isolated from frozen leaf tissue of 1-week-old maize plants (var. Pioneer 33A14) sampled at the beginning of the photoperiod as described by [Carpenter and Simon \(1998\)](#). RT-PCR was performed using gene-specific forward (5'-TACCATGGC CAAGGAGGTGGAC) and reverse (5'-TTTCTACTTGAAGAAG GAGC) primers. PCR products were ligated into the pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA) for sequencing, which was carried out using the M13 forward (5'-CTGGCCGTCGTTTAC) and reverse (5'-GTCATAG CTGTTTCCTG) primers. Resulting *Zmrc1* sequences were aligned and analyzed using the Vector NTI sequence analysis software (InforMax, North Bethesda, MD).

To confirm its activity as activase, a region of the cDNA encoding the putative mature *Zmrc1* protein was subcloned from pCR2.1 TOPO vector into the bacterial expression vector pET23d(+) (Invitrogen, Carlsbad, CA), using restriction enzymes Nco1 and EcoR1 and T4 DNA ligase as described by the supplier (Promega, Madison, WI). The recombinant plasmid was transformed into NovaBlue and BL-21 Star (pLysS) *E. coli* cells (Invitrogen, Carlsbad, CA) for plasmid propagation and protein expression, respectively. Recombinant *Zmrc1* was expressed and purified according to [Salvucci et al. \(2003\)](#), and a spectrophotometric assay ([Salvucci, 1992](#)) verified the activity of this enzyme.

### Purification of wheat and maize pre-EF-Tu from leaf tissue

Leaves from 20-d-old wheat (cultivar Seri-82) and maize (line B-73) plants grown in a greenhouse were collected, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . Total soluble proteins were extracted using an extraction buffer containing 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 10% glycerol, and 1% protease inhibitor cocktail (v/v, Sigma). The homogenates were centrifuged at 12,000g for 20 min. The supernatants were transferred to new tubes and stored at  $-80^{\circ}\text{C}$ . Fifteen milliliter protein extracts were thawed on ice, and concentrated to approximately 1.5 mL using Centricon Plus 20 PL-30 spin columns (Millipore Corp., Bedford, MD) according to the manufacturer's instructions. The concentrated protein extracts were transferred to a 2 mL tube and incubated with anti-maize EF-Tu antibody (100  $\mu\text{L}$ ) ([Bhadula et al., 2001](#)) at  $4^{\circ}\text{C}$  overnight. Two hundred microliter ImmunoPure Immobilized protein A (Pierce Biotechnology, Rockford, IL) was then added, and the mixture was incubated at room temperature for 2 h. The conjugates of chloroplast EF-Tu, anti-EF-Tu antibody, and the

immobilized protein A were precipitated by centrifugation at 2500g at room temperature for 3 min, and washed using an immunoprecipitation buffer containing 25 mM Tris-HCl (pH 7.2). The EF-Tu protein was eluted using the Elution Buffer (Pierce, Rockford, IL). The eluate was neutralized with 1 M Tris-HCl (pH 8.0) at a ratio of 1:10, mixed with glycerol (20% final concentration), and stored at  $-80^{\circ}\text{C}$  until further use. The purified protein was quantitated using RC DC Protein Assay kit (Bio-Rad, Hercules, CA). The purity of pre-EF-Tu preparation was checked using 1-D SDS-PAGE, and the identity was verified by immunoblot analysis ([Rao et al., 2004](#)) using the antibody against maize EF-Tu ([Bhadula et al., 2001](#)). Mass spectrometry was used to verify the identity of purified pre-EF-Tu from wheat leaf tissue ([Rao et al., 2004](#)).

### One-dimensional SDS-PAGE and immunoblotting

One-dimensional SDS-PAGE of purified proteins was carried out according to [Laemmli \(1970\)](#). The SDS-PAGE gels were stained using Coomassie Brilliant Blue R250 (Amersham, Princeton, NJ).

The immunoblot analyses were performed as outlined by [Rao et al. \(2004\)](#). In separate trials, the purified proteins (recombinant maize pre-EF-Tu, recombinant maize rubisco activase, native maize pre-EF-Tu, and native wheat pre-EF-Tu) were resolved on 10% (w/v) polyacrylamide gel with SDS, and then transferred to a PVDF membrane (Bio-Rad, Hercules, CA). The immunoblots with purified pre-EF-Tu were probed using a polyclonal anti-maize EF-Tu antibody ([Bhadula et al., 2001](#)). A previous study has shown that this antibody cross-reacts with wheat EF-Tu ([Ristic et al., 2007](#)). A blot with purified recombinant maize rubisco activase was probed using a monospecific polyclonal anti-tobacco (*Nicotiana tabacum* L.) activase antibody, which has been shown to cross-react with maize ([Crafts-Brandner and Salvucci, 2002](#)) and wheat rubisco activases ([Feller et al., 1998](#)).

### Chaperone assays

Native wheat and maize pre-EF-Tu were tested for possible chaperone activity by monitoring thermal aggregation of recombinant maize activase in the presence or absence of purified wheat or maize pre-EF-Tu as described by [Rao et al. \(2004\)](#). The chaperone assays were also conducted using recombinant proteins with known chaperone activity, namely maize pre-EF-Tu ([Rao et al., 2004](#)) and *E. coli* DnaK ([Diamant et al., 2000](#)). In separate trials, activase (0.75  $\mu\text{M}$ ) was mixed with various increasing concentrations of purified recombinant maize pre-EF-Tu, recombinant *E. coli* DnaK, native maize pre-EF-Tu, and native wheat pre-EF-Tu (as indicated in [Figure 3](#)) in 20 mM Tris-HCl buffer ([Rao et al., 2004](#)). Two controls were used: activase alone and activase mixed with bovine serum albumin (BSA). Samples were incubated at  $25^{\circ}\text{C}$  or  $48^{\circ}\text{C}$  for 45 min in a temperature-controlled micro-multi cell spectrophotometer (Shimadzu, Japan), and activase

stability (aggregation) was estimated by monitoring light scattering at 320 nm during incubation (Rao et al., 2004).

In a separate experiment, the effect of recombinant maize pre-EF-Tu on thermal aggregation of maize activase was analyzed by examining the solubility of activase at high temperature. Both activase (0.75  $\mu$ M) alone and activase mixed with recombinant maize pre-EF-Tu (2  $\mu$ M) were incubated (in Tris-HCl buffer (Rao et al., 2004); 300  $\mu$ L total volume) at 25 °C or 48 °C for 45 min. After incubation, the reaction mixture was centrifuged for 15 min at 15,000g (at 4 °C), and the resulting pellet and supernatant were separated. The pellet was then resuspended in the volume of the Tris-HCl buffer (Rao et al., 2004) that was equal to the volume of the supernatant. The supernatant and the resuspended pellet were analyzed using one-dimensional SDS-PAGE as outlined above. Gel was loaded with equal volumes of protein samples.

## Results and discussion

### Analysis of purified proteins

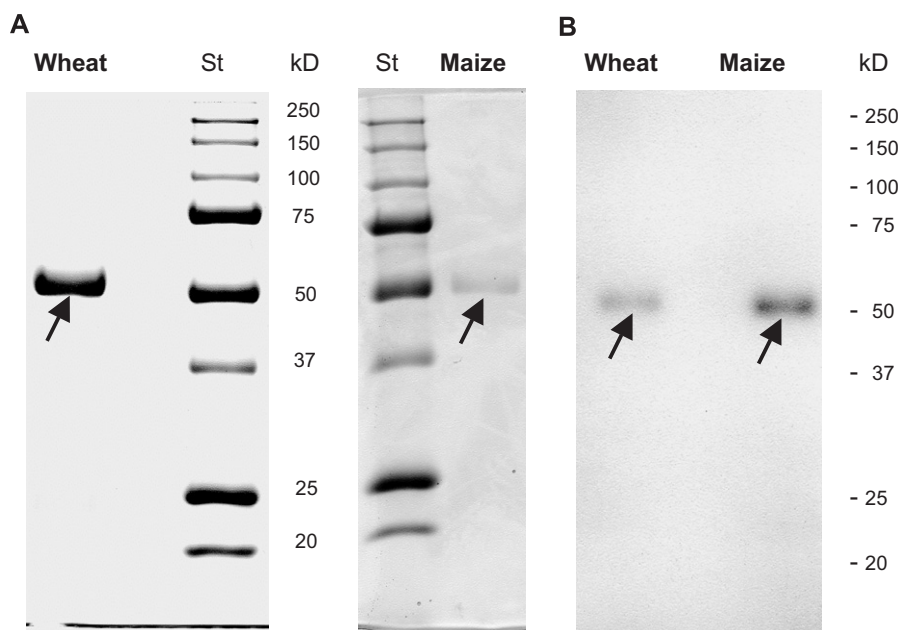
Native wheat and native maize pre-EF-Tu were isolated and purified from leaf tissue of spring wheat cultivar Seri-82 and maize line B-73. Recombinant maize pre-EF-Tu and the mature form of recombinant maize rubisco activase were purified from *E. coli* expressing these proteins. One-dimensional SDS-PAGE analysis of protein prepara-

tions from wheat and maize leaf tissue and from *E. coli* expressing pre-EF-Tu revealed that each purified protein migrated as a single band with a molecular mass of 50–51 kDa, as expected for pre-EF-Tu (Figure 1A and 2A, lane 1). Similar analysis showed that the rubisco activase purified from *E. coli* migrated as a single band with a molecular mass of 43 kDa, as predicted for this protein (Figure 2B, lane 1).

Immunoblot analyses of purified proteins corroborated the results of one-dimensional SDS-PAGE. Immunoblots prepared with purified native wheat, native maize (Figure 1B), and recombinant maize pre-EF-Tu (Figure 2A, lane 2) showed a single band of 50–51 kDa. Likewise, the immunoblot of the purified mature form of maize recombinant activase showed a band of 43 kDa (Figure 2B, lane 2). Mass spectrometry verified the identity of purified native pre-EF-Tu protein from wheat (not shown).

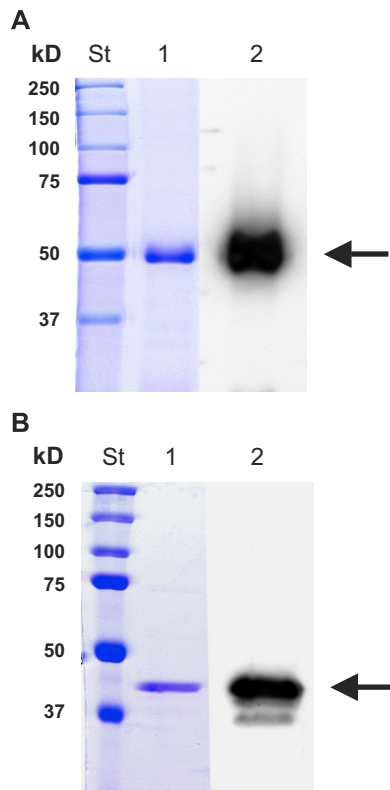
### Protective effect of wheat and maize pre-EF-Tu against thermal aggregation of rubisco activase

Recombinant maize pre-EF-Tu and native wheat and maize pre-EF-Tu were able to protect rubisco activase against thermal aggregation *in vitro*. When heated at 48 °C, activase began to form insoluble aggregates, indicated by an increase in relative light scattering (Figure 3). Activase aggregation,



**Figure 1.** One-dimensional SDS-PAGE gels (A) and immunoblot (B) of purified native wheat and maize pre-EF-Tu. The pre-EF-Tu protein was purified from the leaf tissue of 20-d-old plants. Gels were stained with Coomassie Brilliant Blue R250. The immunoblot was probed with maize anti-EF-Tu antibody (Bhadula et al., 2001). Protein load: A: wheat, 4  $\mu$ g; maize, 1  $\mu$ g; B: wheat, 5 ng; maize, 15 ng. Arrow indicates pre-EF-Tu; St, protein standards.



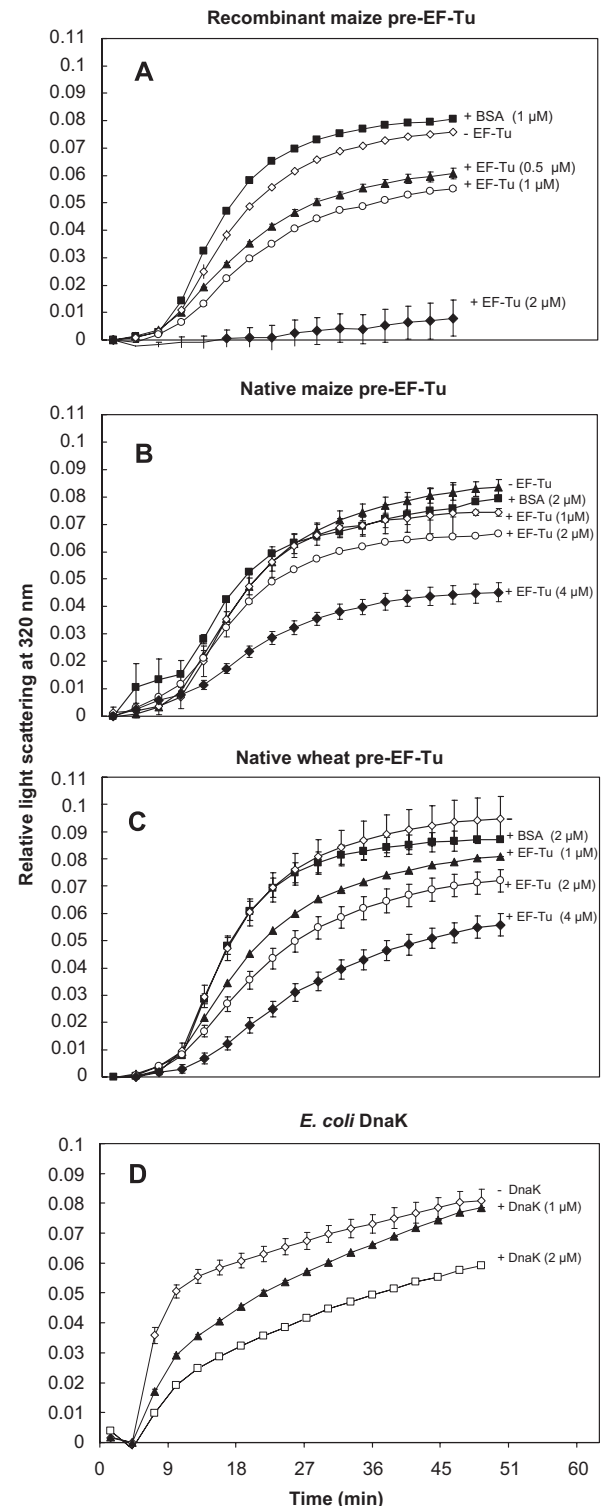


**Figure 2.** One-dimensional SDS-PAGE gels and immunoblots of purified maize recombinant pre-EF-Tu (A) and purified maize recombinant rubisco activase (B). Recombinant proteins were purified from *E. coli* expressing this protein. Panels A and B, lane 1: gels stained with Coomassie Brilliant Blue R250; panel A, lane 2: immunoblot probed with maize anti-EF-Tu antibody (Bhadula et al., 2001); panel B, lane 2: immunoblot probed with anti-tobacco rubisco activase antibody (Feller et al., 1998). Protein load: A: lane 1, 2  $\mu$ g; lane 2, 50 ng; B: lane 1, 1.14  $\mu$ g; lane 2, 40 ng. Arrow indicates purified protein; St, protein standards.

however, was significantly reduced in the presence of various pre-EF-Tu proteins. Recombinant maize pre-EF-Tu almost completely suppressed activase

**Figure 3.** Effect of recombinant maize pre-EF-Tu (A), native maize pre-EF-Tu (B), native wheat pre-EF-Tu (C), and *E. coli* DnaK (D) on thermal aggregation of recombinant maize rubisco activase. In separate trials, rubisco activase (0.75  $\mu$ M) was mixed with increasing concentrations of pre-EF-Tu (EF-Tu). Two controls were used: rubisco activase alone (-EF-Tu) and rubisco activase mixed with bovine serum albumin (BSA). Mixtures (150  $\mu$ L total volume) were incubated at 48  $^{\circ}$ C for 45–50 min. During incubation, samples were monitored for their absorbance at 320 nm, which is indicative of light scattering due to activase aggregation (Salvucci et al., 2001). Data are the mean  $\pm$  SE of two independent experiments. Note that pre-EF-Tu and *E. coli* DnaK protected rubisco activase from thermal aggregation.

aggregation at a pre-EF-Tu:activase molar ratio of 2.7:1 (Figure 3A), and native maize and wheat pre-EF-Tu proteins reduced activase aggregation by nearly 50% at a pre-EF-Tu:activase molar ratio of 5.3:1 (Figure 3B,C). The *E. coli* chaperone DnaK (Diamant et al., 2000) also showed a protective effect against activase aggregation in a



concentration-dependent manner (Figure 3D). In contrast, BSA (1  $\mu$ M, Figure 3A; 2  $\mu$ M, Figure 3B,C) provided no protection against activase aggregation.

The protective effect of pre-EF-Tu in reducing thermal aggregation of rubisco activase was also observed when solubility of activase was examined at high temperature. Activase was highly soluble at room temperature, as revealed by one-dimensional SDS-PAGE analysis of soluble (supernatant) and insoluble (pellet) fractions of centrifuged protein (Figure 4). When activase was heated at 48 °C, most of the protein became insoluble and appeared in the pellet fraction of the protein sample (Figure 4). However, when activase was heated in the presence of recombinant pre-EF-Tu, a considerable portion of the activase protein remained soluble (Figure 4, indicated by arrow), indicating a preventative effect of pre-EF-Tu on activase thermal aggregation.

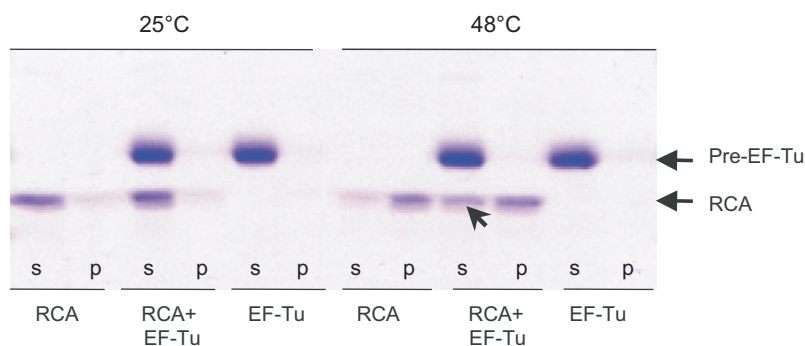
The observation of activase aggregation at high temperature confirms previous reports on thermal sensitivity of this protein (Feller et al., 1998; Salvucci et al., 2001). Immunoblot analysis of protein extracts from detached and heated leaf tissue of wheat and cotton (*Gossypium hirsutum*) showed formation of activase aggregates at elevated temperatures (Feller et al., 1998). Similarly, light-scattering experiments with purified tobacco (*Nicotiana rustica*) activase revealed aggregation of this protein at temperatures of 35 °C and higher (Salvucci et al., 2001).

Our results demonstrating a protective effect of chloroplast pre-EF-Tu against thermal aggregation of activase support previous observations on the chaperone activity of EF-Tu protein. As stated earlier, the recombinant precursor of maize EF-Tu was found to protect citrate synthase and malate

dehydrogenase from thermal aggregation (Rao et al., 2004). Also, bacterial EF-Tu was observed to suppress thermal aggregation of citrate synthase (Caldas et al., 1998).

The present study sheds additional light on the functional properties of chloroplast EF-Tu. It demonstrates that both the native (Figure 3B,C) and recombinant (Rao et al., 2004; Figure 3A) precursor forms of this protein display chaperone activity. Moreover, the ability of pre-EF-Tu from wheat, a C3 species (Akita and Moss, 1972), to protect maize activase from thermal denaturation/aggregation suggests that the chaperone activity of pre-EF-Tu may not be species-specific. Most intriguing, however, is the observation that pre-EF-Tu can protect a heat-labile photosynthetic enzyme, rubisco activase (Salvucci et al., 2001), from thermal aggregation. It is possible that pre-EF-Tu may play a role in protecting the photosynthetic apparatus during high-temperature stress. Further studies are needed to investigate this hypothesis.

The ability of chloroplast pre-EF-Tu to protect rubisco activase from thermal aggregation supports the hypothesis that the native mature form of this protein plays a role in heat tolerance by acting as a molecular chaperone. Native EF-Tu is localized in chloroplast stroma (Momcilovic and Ristic, 2004) and may protect chloroplast stromal proteins, including activase, from thermal aggregation. This hypothesis is supported by Momcilovic and Ristic (2004) and Ristic et al. (2004), who observed a negative correlation between endogenous levels of EF-Tu and thermal aggregation of chloroplast stromal proteins. Previous studies have also shown that whole chloroplasts from maize lines that have higher levels of EF-Tu are more heat stable than those from low-level EF-Tu lines (Ristic and Cass,



**Figure 4.** Effect of recombinant pre-EF-Tu (EF-Tu) on solubility of rubisco activase at high temperature. In separate trials, rubisco activase alone (RCA) and rubisco activase mixed with pre-EF-Tu were incubated at 25 °C or 48 °C for 45 min. After incubation, soluble (s, supernatant) and insoluble/aggregated (p, pellet) protein fractions were separated by centrifugation and were analyzed using one-dimensional SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R250. An increase in the amount of soluble activase was noted (indicated by arrow) when it was heated at 48 °C in the presence of pre-EF-Tu.

1992, 1993; Ristic et al. 1996). In addition, a recent study has shown that chloroplasts from a group of wheat cultivars that accumulate more EF-Tu under heat stress conditions display better thermal stability than chloroplasts from a group of cultivars that accumulate less EF-Tu (Ristic et al., 2007).

It could be argued that the chaperone activity of pre-EF-Tu may be specific to this precursor protein, rather than the mature form of EF-Tu, because of the presence of a chloroplast targeting sequence (Bhadula et al., 2001). It is formally possible that the mature form of EF-Tu may not exhibit chaperone properties. Although we do not completely rule out this possibility, there is evidence to suggest that the native EF-Tu displays chaperone activity. The precursor of EF-Tu has the ability to bind GDP (Rao et al., 2004), as does native EF-Tu (Stanzel et al., 1994), an indication that the targeting sequence does not affect the functional properties of this protein. In addition, the amino acid sequence of eukaryotic EF-Tu is strikingly similar to that of bacterial EF-Tu (Bhadula et al., 2001), which is known to display chaperone activity (Caldas et al., 1998). Moreover, the predicted two-dimensional (SCRATCH servers; <http://www.igb.uci.edu/tools/scratch/>) and three-dimensional (Schwede et al., 2003) structures of pre-EF-Tu are very similar to that of native EF-Tu, implying that the functional properties of pre-EF-Tu and native EF-Tu may be equivalent.

In conclusion, the results of this study indicate that the native precursor of both wheat and maize chloroplast EF-Tu displays chaperone activity, as it reduced thermal aggregation of rubisco activase *in vitro*. To our knowledge, this is the first demonstration of chaperone activity of native pre-EF-Tu and the first observation of thermal protection of a photosynthetic enzyme, rubisco activase, by this putative chaperone. The results support the hypothesis that EF-Tu plays a role in heat tolerance by acting as a molecular chaperone. Further studies to determine the role of native EF-Tu in protecting photosynthetic enzymes during periods of heat stress in plants are warranted.

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